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Varicella-Zoster Virus ORF57, Unlike Its Pseudorabies Virus UL3.5 Homolog, Is Dispensable for Viral Replication in Cell Culture

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Varicella zoster virus (VZV) encodes five genes that do not have homologs in herpes simplex virus. One of these genes, VZV ORF57, is predicted to encode a protein containing 71 amino acids. Antibody to ORF57 protein immunoprecipitated a 6-kDa protein in the cytosol of VZV-infected cells. Although the homolog of VZV ORF57 in pseudorabies virus, UL3.5, is critical for viral egress and growth in cell culture, VZV unable to express ORF57 replicated to titers similar to those seen with parental virus. Thus VZV ORF57 has a different role in viral replication than its pseudorabies virus homolog.

INTRODUCTION

Varicella-zoster virus (VZV) is a member of the alphaherpesvirus subfamily. This subfamily is further divided into the genus *Simplexvirus*, which includes herpes simplex virus (HSV) and herpesvirus simiae, and *Varicellovirus*, which includes VZV, equine herpesvirus type 1 (EHV-1), EHV-4, bovine herpesvirus type 1 (BHV-1), and pseudorabies virus (PRV).

VZV encodes at least 69 unique genes, and all except five of these genes have homologs in HSV (Cohen and Straus, 1996). Three of the five genes, ORFs 1, 13, and 32, have been shown to be dispensable for replication of VZV *in vitro*. ORF1 encodes a membrane protein (Cohen and Seidel, 1995), whereas ORF32 encodes a phosphoprotein that is posttranslationally modified by the VZV ORF47 kinase (Reddy *et al.,* 1998). ORF13 encodes the viral thymidylate synthetase (Cohen and Seidel, 1993). The other two VZV proteins that do not have HSV homologs have not been studied.

VZV ORF57 is predicted to encode a 71-amino-acid protein containing hydrophilic and basic residues (Davison and Scott, 1986). Although VZV ORF57 does not have a homolog with HSV, it does share positional and limited sequence homology with other *Varicellovirus* proteins. These include EHV-1 gene 59 (Telford *et al.,* 1992), EHV-4 gene 59 (Telford *et al.,* 1998), PRV UL3.5 (Dean *et al.,* 1993), BHV-1 UL3.5 (Khattar *et al.,* 1995), and infectious laryngotracheitis virus UL3.5 proteins (Fuchs and Mettenleiter, 1996). Although VZV ORF57 protein is predicted to be 71 amino acids in length, the other proteins range in size from 72 amino acids for infectious laryngotracheitis virus UL3.5 to 220 amino acids for PRV UL3.5. These proteins have sequence homology in their first 50 amino acids (Khattar *et al.,* 1995) and contain a large number of basic amino acids with isoelectric points ranging from 10 to 13. BHV-1 UL3.5 is a virion protein associated with the tegument or envelope whose role in virus replication is unknown (Schikora *et al.,* 1998). In contrast, PRV UL3.5 encodes a nonstructural protein that is critical for viral egress (Fuchs *et al.,* 1996).

Here we show that VZV ORF57 encodes a 6-kDa protein present in the cytosol of virus-infected cells. Unlike its PRV counterpart, deletion of VZV ORF57 does not impair growth of the virus in cell culture.

RESULTS AND DISCUSSION

To verify that ORF57 is expressed in VZV-infected cells, rabbit antibodies were made to a fusion protein derived from VZV ORF57. Immunoprecipitation of [^{35}S]methionine-labeled cells showed a 6-kDa protein from VZV-infected cells using antisera to VZV ORF57 (Fig. 1A). A similar-sized protein was not present in uninfected cells. Immunoprecipitations of [^{32}P]orthophosphoric acid-radiolabeled VZV-infected cells were performed to determine whether the ORF57 protein is phosphorylated. Antibody to ORF57 protein did not immunoprecipitate a phosphoprotein from VZV-infected cells, whereas antibody to gE detected the phosphorylated glycoprotein (data not shown).

Cytosolic and membrane fractions were prepared from radiolabeled VZV-infected cells to determine where ORF57 protein is located in infected cells. Immunoprecipitation with antibody to ORF57 protein showed that the protein was located in the cytosolic fraction of infected cells but not in the membrane fraction (Fig. 2). As a control for separation of the cellular fractions, VZV gE localized to the membrane but not the cytosolic fraction.

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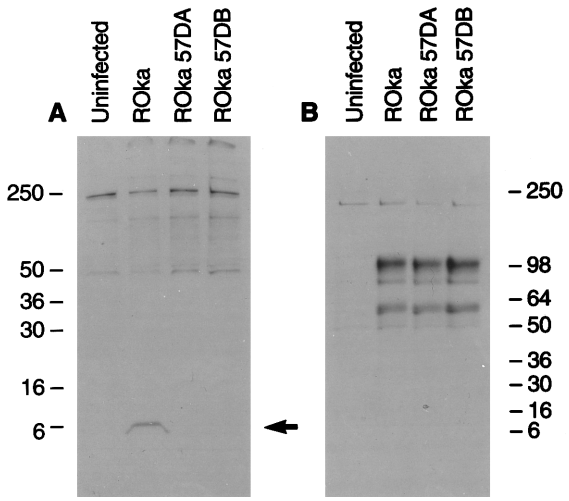

FIG. 1 Characterization of ORF57 protein from VZV-infected cells. (A) Antibody to ORF57 protein immunoprecipitates a 6-kDa protein in VZV ROka-infected cells (arrow) but not in ROka57DA- or ROka57DB-infected cells. (B) Cells infected with VZV ROka, ROka57DA, or ROka57DB express proteins of 60–100 kDa that react with monoclonal antibody to VZV gE. Numbers refer to molecular weight of proteins in kilodaltons.

Unlike VZV ORF57, PRV UL3.5 is located in the membrane-plus-microsome fraction of virus-infected cells (Fuchs *et al.,* 1996).

To determine whether ORF57 is essential for growth of VZV *in vitro*, cells were transfected with cosmids *Not*I A, *Mst*II B, *Mst*II A-57DA, or *Mst*II A-57DB and plasmids pNotI B and pCMV62. Cytopathic effects, indistinguishable from those seen with parental VZV, were present in cells transfected with the ORF57 deletion mutant cosmids. Virion DNA was prepared from cells infected with VZV ROka and ROka57D, and Southern blots were performed to verify that the genomes had the expected configurations. Digestion of DNA from VZV ROka57D with *Eco*RI showed restriction fragments that were identical to those seen with the parental virus (Fig. 3A). Digestion of DNA from ROka with *Sph*I showed a 18-kb band, whereas DNA from ROka57D had a 16-kb band due to the deletion in ORF57 (Fig. 3B).

To verify that cells infected with ROka57D were unable to express ORF57 protein, infected cells were radiolabeled and lysates were immunoprecipitated with antibody to the proteins. Although cells infected with ROka expressed a 6-kDa protein that reacted with antibody to ORF57 protein, ROka57DA- and ROka57DB-infected cells did not produce a similar-size protein (Fig. 1A). To ensure that the absence of expression of ORF57 protein was not due to the lack of VZV gene expression, immunoprecipitations were performed from cells infected with the ORF57 deletion mutants with antibody to gE. Cells infected with the mutants expressed VZV gE (Fig. 1B).

Melanoma cells were infected with cells containing the ORF57 mutant virus, and the plaque sizes were measured to determine whether the absence of ORF57 affects the growth of VZV *in vitro*. The mean size of plaques (± the standard deviation) produced by the ROka57DA (0.87 ± 0.21 mm) was not statistically different from the size of plaques produced by ROka (0.80 ± 0.16 mm) in melanoma cells ($P = .21$, Tukey's multiple comparison test). The size of plaques from the ORF57 mutant and ROka were similar in U2OS osteosarcoma cells (0.44 ± 0.09 and 0.38 ± 0.04 mm, respectively) and in schwannoma cells (0.60 ± 0.07 and 0.72 ± 0.08, respectively).

To further verify that the ORF57 deletion mutants were not impaired for growth *in vitro*, melanoma cells were infected with the ORF57 mutants and the titer of virus was determined at different time points. VZV ROka57D grew to titers similar to those seen in cells infected with the parental (ROka) virus (Fig. 4).

Although the VZV ORF57 mutant was not impaired for growth in cell culture, a PRV UL3.5 mutant that truncates the protein after the first 10 amino acids was severely impaired for growth *in vitro* (Fuchs *et al.,* 1996). The PRV UL3.5 deletion mutant was blocked for the development and release of virions from infected cells and required a complementing cell line to produce plaques.

Although BHV-1 UL3.5 and PRV UL3.5 proteins show limited sequence identity (26%) and difference in size, the BHV-1 protein can complement the PRV protein (Fuchs *et al.,* 1997). These findings, along with the observation that other alphaherpesviruses have UL3.5 homologs led Fuchs *et al*. (1997) to postulate that "members of this gene family might contribute to the same general step of alphaherpesvirus maturation and egress." Our observation that VZV ORF57, the homolog of PRV UL3.5, is fully dispensable for repli-

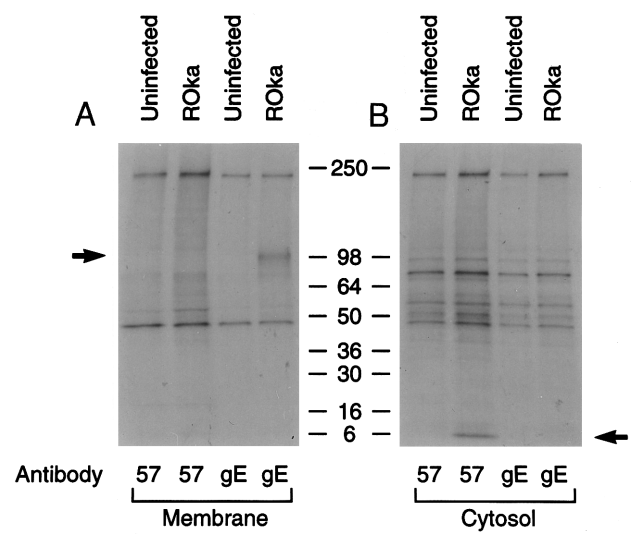

FIG. 2. VZV ORF57 is a cytosolic protein. Cells infected with VZV ROka were radiolabeled with [^{35}S]methionine, and membrane (A) and cytosolic (B) fractions were prepared. An aliquot of each fraction was immunoprecipitated using antibody to ORF57 (lanes 1 and 2) or VZV gE (lanes 3 and 4) proteins. VZV ORF57 protein is detected only in the cytosolic fraction of cells infected with VZV (arrow), whereas gE is present in the membrane fraction (arrow). Numbers refer to molecular weight of proteins in kilodaltons.

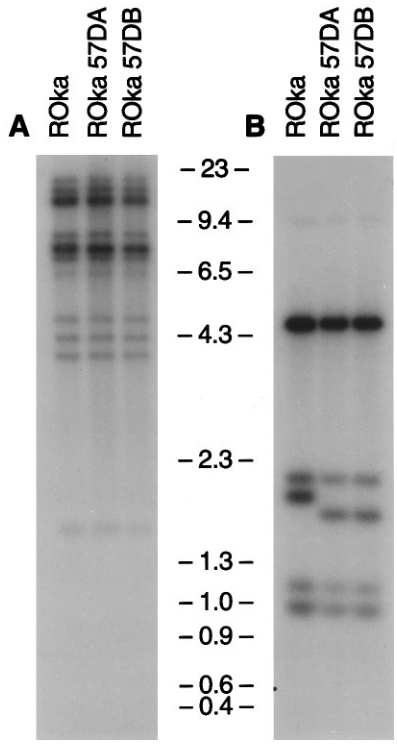

FIG. 3. Southern blot of virion DNAs from VZV ORF57 deletion mutants. (A) Virion DNA digested with *Eco*RI and probed with all four radiolabeled cosmids shows no rearrangement of the genome. (B) Digestion of ROka57D with *Sph*I followed by hybridization with a probe from the ORF57 region of VZV shows that a 18-kb band in ROka is reduced in size to 16 kb in ROka57D due to the deletion in ORF57. Numbers refer to molecular weights in kilobase pairs.

cation in cell culture indicates that the VZV gene differs substantially from its PRV counterpart. In contrast to PRV, VZV is highly cell associated *in vitro* and little or no cell-free virus is released into the medium. Thus, future ultrastructural studies will be required to determine the role of the VZV ORF57 protein in virion maturation and spread *in vitro*.

MATERIALS AND METHODS

Cells and viruses

MeWo (human melanoma) cells were used for transfections and preparation of virus stocks. Recombinant viruses were derived from cosmids corresponding to the attenuated Oka strain of VZV.

Plasmids and cosmids

VZV cosmids *Not*I A, *Mst*II A, and *Mst*II B and plasmid *Not*I B contain the entire VZV genome (Fig. 5). To produce VZV with a deletion in ORF57, the *Mst*IIA cosmid was cut with *Nhe*I, which cuts at VZV nucleotides 91,047 and 100,741, and the 9.7-kb fragment was inserted into the *Nhe*I site of plasmid Litmus 38 (New England Biolabs, Beverly, MA) in which the *Dra*III site had been ablated previously. Because *Dra*III recognizes CACNNNGTG, the *Dra*III site of plasmid Litmus 38 was ablated, and two new *Dra*III sites, corresponding to the *Dra*III sites near ORF57 (VZV nucleotides 98,632 and 99,818) were inserted. Oligonucleotides CTAGTCCACGTTGTGGA and AGCTTCCACAACGTGGA were used to insert the first *Dra*III site at the *Spe*I and *Hin*dIII sites of the plasmid, and oligonucleotides GATCCCCACGGGGTGCG and AATTCGCACCCCGTGGG were used to insert the second *Dra*III site at the *Bam*HI and *Eco*RI sites of the plasmid. The resulting plasmid, pLit38-*Dra*III-2, was cut with *Dra*III, and the 12-kb *Dra*III fragment containing ORF57 from the *Nhe*I plasmid was inserted. The resulting plasmid was cut with AgeI and BsiWI (which cut at VZV nucleotides 99,450 and 99,616), and two oligonucleotides, CCGGTTACGTTCTC and GTACGAGAACGTAA were annealed and ligated into the *Age*I and *Bsi*WI sites of the plasmid. The latter oligonucleotides were used to restore the 3′ carboxyl terminus of ORF58, which overlaps the first 17 nucleotides of ORF57. The sequence of the inserted oligonucleotides was confirmed in two independent plasmid clones. The two clones then were cut with *Dra*III, and the mutated DNAs were inserted into the *Nhe*I plasmid. The latter then were cut with *Nhe*I, and the ORF57 mutant DNA was inserted into cosmid *Mst*II A to produce cosmids *Mst*II A-57DA and *Mst*II A-57DB. These cosmids have a 152-nucleotide deletion that begins 20 nucleotides after the start of ORF57 and ends 41 nucleotides before the stop codon of ORF57.

Transfections

Cosmids were digested with *Not*I or *Bsu*36I to linearize the DNAs. To produce VZV with a deletion in ORF57, cells were transfected with 1 μg of cosmid *Not*I A, *Mst*II B, 0.5 μg of cosmid *Mst*II A-57D, 1 μg of plasmid *Not*I B, 50 ng of plasmid pCMV62, and 2 μg of salmon sperm DNA. Transfection of MeWo cells was performed as previously described (Cohen and Seidel, 1993).

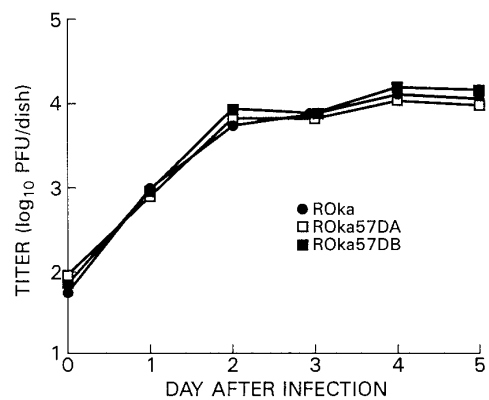

FIG. 4. Growth of VZV ROka, ROka57DA, and ROka57DB in Mewo cells. MeWo cells were inoculated with VZV-infected cells, and at various times after infection the cells were harvested and the titer of virus was determined in MeWo cells.

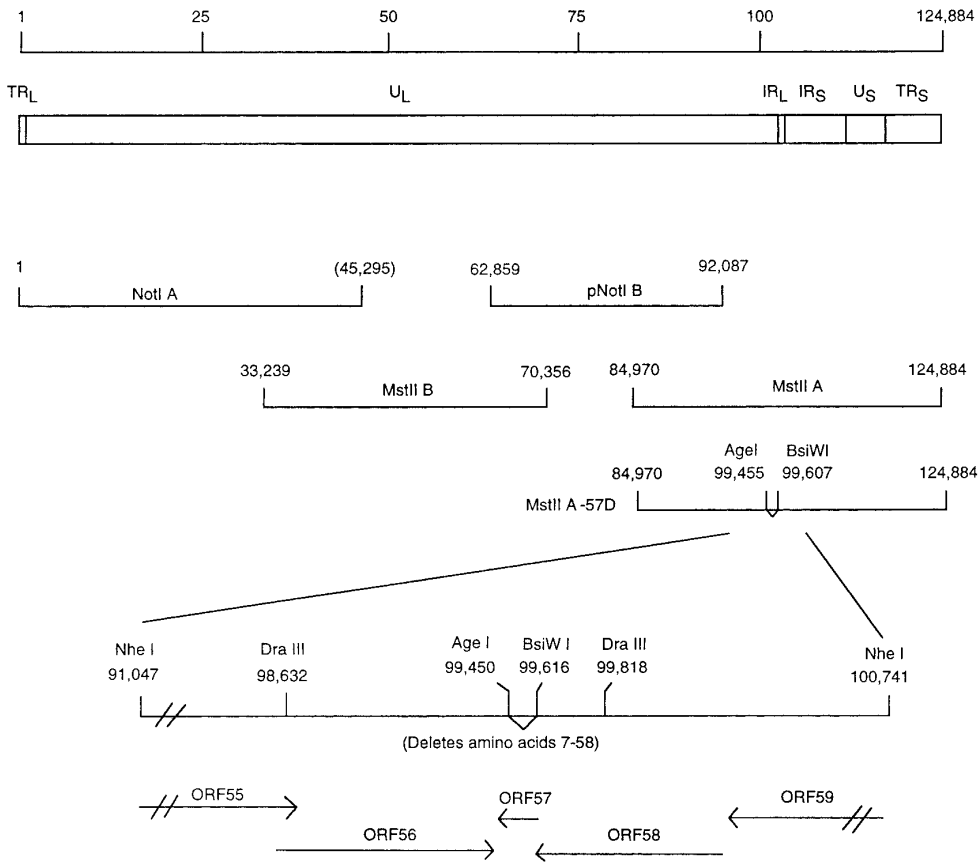

FIG. 5. Construction of recombinant VZV with a deletion in ORF57. The VZV genome is 124,884 bp in length (line 1) and contains unique long (U_L), unique short (U_S), terminal repeat (T_R), and internal repeat (IR) DNA sequences (line 2). *Not*I and *Mst*II restriction fragments used to generate parental VZV are shown (lines 3 and 4). Cosmid *Mst*II A-57D (line 5) has a deletion beginning at codon 7 and ending at codon 58. Numbers for cosmid *Mst*II A-57D indicate the site of the deletion. This site is generated by oligonucleotides inserted at the AgeI and BsiWI restriction endonuclease sites. Two identical, independent cosmid clones, *Mst*IIA-57DA and *Mst*IIA-57DB, were used to construct viruses with a deletion in ORF57. Restriction endonuclease sites used for construction of cosmid *Mst*II A-57D (line 6) and location of VZV open reading frames in this region (line 7) are shown.

Southern and Northern blots

VZV DNA was purified from nucleocapsids, cut with *Eco*RI or *Sph*I, fractionated on agarose gels, and transferred to nylon membranes. The four DNA cosmids spanning the entire VZV genome were radiolabeled with [^{32}P]dCTP and hybridized to the immobilized VZV DNA. An *Nhe*I fragment, containing VZV nucleotides to 91,047–100,741, was radiolabeled and used to demonstrate the ORF57 mutation.

Growth characteristics of recombinant VZV

Growth curves for recombinant VZV were performed by infecting MeWo cells with cells containing about 100 pfu of VZV. At days 1, 2, 3, 4, and 5 after infection, the cells were harvested, and serial dilutions were used to inoculate uninfected MeWo cells. Plaques were stained and counted 7 days after infection.

Generation of ORF57 fusion proteins

The coding region of VZV ORF57, from codons 11–71, was amplified from VZV cosmid DNA by PCR. The first primer contained a *Bam*HI site followed by VZV nucleotides 99,576–99,596 (CGCGGATCCAATGCCAGCGTTGCCACGCCG), and the second primer contained an *Eco*RI site followed by VZV nucleotides 99,414–99,434 (CGCGAATTCACGTTGATGAGCCTTGCAGGT). The amplified DNA was digested with *Bam*HI and *Eco*RI and inserted into plasmid pGEX-2T, and the sequence of the junction of the ORF57 DNA and pGEX-2T DNA was confirmed.

Escherichia coli containing the plasmid expressing the GST-ORF57 fusion protein were grown in 2XYT medium with 2% glucose and ampicillin, and IPTG was added. The bacteria were lysed by sonication, and the GST-ORF57 fusion protein was bound to glutathione–Sepharose, washed extensively, eluted with reduced glutathione, and dialyzed.

Antibodies, immunoprecipitation, *in vitro* translation, and cell fractionation studies

Rabbits were immunized once with 150 μg of GST-ORF57 fusion proteins in complete Freund's adjuvant and two additional times in incomplete Freund's adju-

vant. Antiserum was obtained and absorbed four times with lysates of uninfected MeWo cells.

VZV-infected and uninfected cells were radiolabeled with [^{35}S]methionine or [^{32}P]orthophosphoric acid and lysed. The supernatant was incubated with rabbit antibody to ORF57 protein or monoclonal antibody to VZV gE (Chemicon, Temecula, CA) followed by protein A–Sepharose. Immune complexes were fractionated on SDS–polyacrylamide gels. Membrane and cytosolic fractions from VZV-infected MeWo cells were prepared as previously described (Cohen and Seidel, 1995).

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